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10. Rats were anesthetized and transcardiacally perfused as described (*7*, *8*). Cryosections (35 μm) were sequentially incubated with antibodies against FRAs (*7*) (dilution, 1:4000), and tyrosine hydroxylase (TH; dilution 1:500) (Chemicon) was used to stain DA terminals. Detection was performed with avidin-biotin-peroxidase complex (Vector Laboratories), followed by diaminobenzidine for TH and nickel enhancement for FRA signals. The number of positive nuclei was measured within a squared field area of 270 × 270 μm with the use of the IBAS image analyses system (Zeiss, Germany). Each area was measured in four adjacent coronal sections, and the results were averaged. For details, see M. Zoli *et al.*, *Neurochem. Int.* **16**, 383 (1990).
11. All rats were kept on a restricted diet regime (85% of the normal body weight). Nicotine (0.03 mg per kilogram of body weight) was delivered in a volume of 22 μl per infusion, cocaine (0.25 mg/kg) at 100 μl per infusion, and saline at 22 μl per infusion. These doses were selected among those that can maintain stable self-administration in rats [B. C. Caine and G. F. Koob, *J. Pharmacol. Exp. Ther.* **270**, 209 (1994)] (*4*, *5*). No claim for equipotency of the two drugs was made because direct comparison of the reinforcing effects of cocaine and nicotine requires further studies. Thus, no direct statistical comparison was planned between nicotine and cocaine self-administration groups. Stable baseline performance was defined when the rate of lever pressing between three consecutive sessions did not vary more than 20%.
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14. Acute administration of nicotine in saline rats during the last session did not change AP-1 binding in any brain region when compared with that in saline rats (*17*).
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19. When the mutant oligonucleotide (GCC GCA ATG TCA GAC TAG CGG G) was used, no bands were detected (*17*).
20. We thank M. Iadarola for providing the antibody to FRAs; R. A. North for discussing the manuscript; S. Catsicas, J. Knowles, D. Trist, and E. Ratti for constant support; and S. DeVevey and E. Valerio for excellent technical assistance. All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals (NIH). Part of these data was presented at the Society for Neuroscience meeting [C. Chiamulera *et al.*, *Soc. Neurosci. Abstr.* **21**, 722 (1995)].

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Interaction of the Thiol-Dependent Reductase ERp57 with Nascent Glycoproteins

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Calnexin and calreticulin interact specifically with newly synthesized glycoproteins in the endoplasmic reticulum (ER) and function as molecular chaperones. The carbohydrate-specific interactions between ER components and glycoproteins synthesized in isolated canine pancreatic microsomes were analyzed using a cross-linking approach. A carbohydrate-dependent interaction between newly synthesized glycoproteins, the thiol-dependent reductase ERp57, and either calnexin or calreticulin was identified. The interaction between ERp57 and the newly synthesized glycoproteins required trimming of the N-linked oligosaccharide side chain. Thus, it is likely that ERp57 functions as part of the glycoprotein-specific quality control machinery operating in the lumen of the ER.

The lumen of the ER contains a number of molecular chaperones that assist in the later stages of protein biosynthesis and folding (*1*, *2*). A number of studies have highlighted specific interactions between newly synthesized glycoproteins and the putative chaperones calnexin and calreticulin (*3*–*6*). The binding of calnexin and calreticulin to newly synthesized proteins is normally characterized by a specific requirement for correctly processed, asparagine-linked (N-linked), carbohydrate side chains. In combination with uridine 5′-diphosphate (UDP)–glucose:glycoprotein glucosyltransferase (*7*), calnexin and calreticulin are thought to mediate a quality control cycle for newly synthesized glycoproteins (*2*, *8*, *9*). The function of this cycle is to ensure that only correctly folded and assembled proteins exit the ER and gain access to later compartments of the secretory pathway (*2*, *10*).

Here, we used model substrates derived from the secretory protein preprolactin (PPL) (*11*) to determine the effect of N-linked glycosylation on the interactions between newly synthesized polypeptides and ER proteins. When the PPL92.CHO transcript was translated in vitro in the presence of canine pancreatic microsomes, a glycosylated 62–amino acid prolactin fragment (PL62.CHO) was generated. In contrast, translation of the PPL92.Con transcript generated a nonglycosylated 62–amino acid fragment (PL62.Con). The interactions between both PL62.CHO and PL62.Con and ER components were analyzed with the use of the membrane-permeable cross-linking reagent succinimidyl 4-(*N*-maleimidomethyl) cyclohexane carboxylate (SMCC), a heterobifunctional reagent that principally cross-links lysines to cysteines. Two groups of cross-linking partners could be identified: (i) ER proteins that interacted with both glycosylated and nonglycosylated polypeptides, exemplified by protein disulfide isomerase (PDI) (Fig. 1A); and (ii) ER proteins that interacted only with glycosylated polypeptides. Calnexin and calreticulin, which interact principally with glycoproteins, were observed to cross-link to PL62.CHO (Fig. 1B) but not to PL62.Con (Fig. 1A). A weaker 160-kD product (Fig. 1B) presumably represented a ternary cross-linking adduct of calnexin, PL62.CHO, and a third unidentified component.

In addition to antisera that recognized calnexin, calreticulin, and PDI, a number of antisera to other ER luminal proteins were screened for immunoprecipitation of glycosylation-dependent (that is, PL62.CHO-specific) cross-linking products (*12*). We were able to identify ERp57 (*13*), a thiol-dependent reductase (*14*) and putative cysteine protease (*15*), as a strong cross-linking partner of PL62.CHO but not of PL62.Con (compare Figs. 1A and 1B). This result suggested that, like calnexin and calreticulin, ERp57 interacts specifically with glycoproteins.

When immunoprecipitation was performed under "native" conditions (*6*), a 69-kD cross-linking product was coprecipitated with both calnexin and calreticulin (Fig. 1B). This 69-kD product was absent when samples were denatured with SDS before immunoprecipitation (Fig. 1B). The glycosylated PL62.CHO product had an apparent molecular mass of 9 kD (*11*), which implied that a 60-kD cross-linking partner was coprecipitated with the calnexin and calreticulin cross-linking products. A similar 60-kD calnexin-associated protein, denoted CAP-60, coprecipitates with adducts of the Glut-1 glucose transporter and calnexin (*6*).

The 69-kD cross-linking product obtained with PL62.CHO had a similar mobility to those obtained with both PDI and ERp57 (Fig. 1B). To establish whether CAP-60 was actually one of these components, we performed sequential immunopre-

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cipitations. After native immunoprecipitation, samples were denatured with SDS and reprecipitated. The 97- and 160-kD calnexin-derived cross-linking products were reprecipitated with anti-calnexin serum, but CAP-60 was no longer observed and could not be reprecipitated with anti-PDI serum (Fig. 2A). Likewise, the 71-kD calreticulin cross-linking product was reprecipitated by the anti-calreticulin serum, but no products were recognized by anti-PDI serum (Fig. 2A). In a control experiment, anti-PDI serum worked well in sequential immunoprecipitations of PDI cross-linking products (Fig. 2A). A similar experiment showed that the 69-kD cross-linking product, which associated with both calnexin and calreticulin, was reprecipitated with antibodies specific for ERp57 (Fig. 2B) (*16*). Thus, CAP-60 was in fact ERp57 and interacted with glycoproteins in combination with calnexin and calreticulin.

To investigate further the interaction between ERp57 and glycoproteins, we analyzed two authentic glycosylated secretory proteins (*17*) by cross-linking. Both yeast pro–α factor and human interferon-γ (IFN-γ) were found to be cross-linked to ERp57 and calreticulin (Fig. 3, B and C). Thus, ERp57 could be cross-linked to a variety of glycoproteins.

Calnexin and calreticulin interact preferentially with glycoprotein substrates that have been enzymatically processed to bear the monoglucosylated form of the N-linked carbohydrate side chain (*2*). To determine the role of glucose trimming in promoting the interaction of glycoproteins with ERp57, we used castanospermine to specifically inhibit the glucosidases responsible for this processing (*9*). The efficacy of the castanospermine treatment was established by comparing the mobility of the glycoproteins with and without the treatment (*5*). In each case, castanospermine caused a reduction in the mobility of the glycosylated proteins due to the increase in the number of glucose residues present on the carbohydrate side chain (Fig. 3D). The interactions of calnexin (Fig. 3A), calreticulin (Fig. 3, A to C), and ERp57 (Fig. 3, A to C) were all substantially inhibited by castanospermine treatment. In contrast, cross-linking to PDI

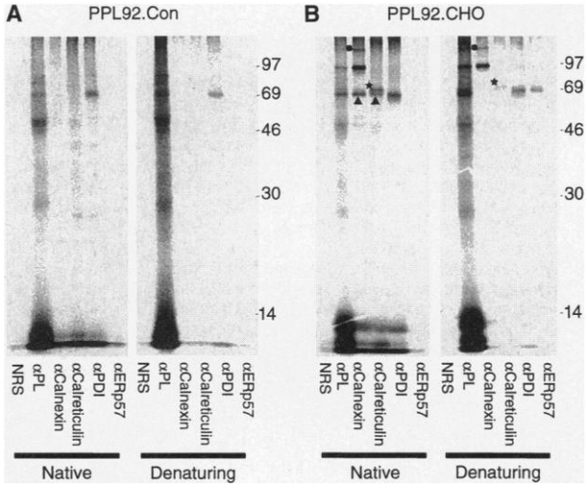

Fig. 1. Glycosylation of imported polypeptides leads to specific interactions with ER proteins. PPL92.CHO and PPL92.Con mRNAs (*11*) were translated in a wheat germ lysate supplemented with canine pancreatic microsomes and [^{35}S]methionine (*20, 21*). The microsomes were isolated and incubated with 1 mM SMCC (*19, 22*). (**A**) PL62.Con cross-linking products were immunoprecipitated before (native) or after (denaturing) denaturation with 1% SDS (*6*). Affinity-purified anti-ERp57 (*23*), a nonrelated control serum (NRS), and rabbit antisera (α) specific for prolactin (PL), calnexin, calreticulin, and PDI were used. (**B**) PL62.CHO cross-linking products were analyzed exactly as above. Samples were resolved by electrophoresis on 12% SDS-polyacrylamide gels and visualized with a Fujix BAS-2000 bioimaging system and software. The apparent molecular masses of calnexin and calreticulin are 88 and 60 kD, respectively. The prominent 97-kD cross-linking product immunoprecipitated by the anti-calnexin serum is a PL62.CHO-calnexin adduct. Stars denote the 71-kD calreticulin cross-linking product, the arrowheads show the CAP-60 product, and the solid circle is a minor 160-kD calnexin-containing adduct. In this and subsequent figures, molecular size markers (in kilodaltons) are at the sides of lanes.

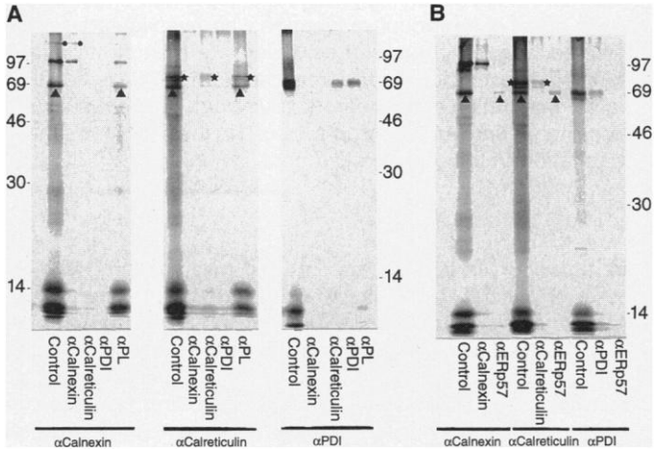

Fig. 2. Identification of CAP-60 by sequential immunoprecipitation using PPL92.CHO mRNA. (**A**) After immunoprecipitation with anti-calnexin, anti-calreticulin, or anti-PDI sera, samples were denatured with SDS and reprecipitated with antibodies specific for calnexin, calreticulin, PDI, or the nascent chain (PL). Control lanes show products obtained after the first round of immunoprecipitation. (**B**) After immunoprecipitation with anti-calnexin, anti-calreticulin, and anti-PDI sera, samples were denatured with SDS and reprecipitated with sera specific for calnexin, calreticulin, or PDI, or with affinity-purified anti-ERp57 (*23*). Control lanes show products obtained after the first round of immunoprecipitation. The symbols in the lanes have the same meanings as in Fig. 1.

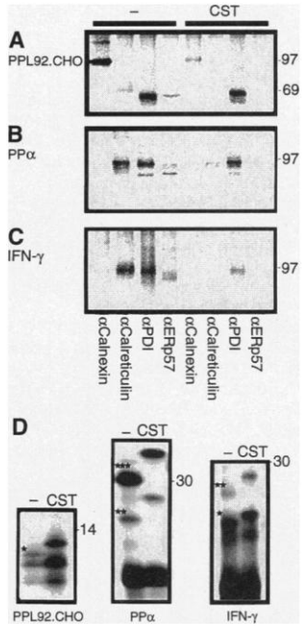

Fig. 3. The interaction of ERp57 with glycoproteins requires trimming of the oligosaccharide side chains. mRNAs encoding PPL92.CHO, *S. cerevisiae* prepro–α factor (PPα), and human IFN-γ (*17*) were translated in the presence of canine pancreatic microsomes, and imported polypeptides were cross-linked to interacting components with SMCC (*22*). The glucosidase inhibitor castanospermine (CST) was included at 1 mM during the translation reaction as indicated (–, no CST added). (**A** to **C**) Cross-linking products were denatured with 1% SDS and then analyzed by immunoprecipitation with anti-calnexin, anti-calreticulin, or anti-PDI sera or affinity-purified anti-ERp57 (*23*). (**D**) Castanospermine treatment inhibits glucose trimming, resulting in a reduced mobility for each glycoprotein examined. The major glycosylated forms of each polypeptide after trimming are indicated by stars; the number of stars indicates the number of N-linked carbohydrate side chains present (*24*).

was either unaffected (Fig. 3, A and B) or only partially inhibited (50%) (Fig. 3C).

Thus, like calnexin and calreticulin, ERp57 binding required glucose trimming of the N-linked carbohydrate side chains. We propose that ERp57 functions in combination with calnexin and calreticulin as a molecular chaperone of glycoprotein biosynthesis. We observed a time-dependent decrease in the amount of the PL62.CHO cross-linking products with calnexin, calreticulin, and the associated ERp57 (*18*). This suggested that the interaction between ERp57 and nascent glycoproteins was transient, like other molecular chaperone-substrate interactions (*3*, *5*, *19*). We believe a specific modulation of glycoprotein folding could be achieved by coupling the lectin-like properties of calnexin and calreticulin (*2*) with the thiol-dependent reductase activity of ERp57 (*14*).

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11. PPL92.Con encodes the NH_2-terminal 92 amino acids of the wild-type bovine preprolactin sequence followed by a stop codon. PPL92.CHO encodes the NH_2-terminal 88 amino acids of the wild-type sequence, followed by 4 amino acids incorporating a consensus site for N-linked glycosylation, and finishing with a stop codon. PPL92.Con was prepared by means of the polymerase chain reaction; PPL92.CHO was prepared by the ligation of a linker to the 3′ end of Pvu II–cut PPL cDNA. The amino acid sequence of the COOH-terminal 10 residues of each polypeptide is as follows: PPL92.Con: Met-Ala-Leu-Asn-Ser-Cys-His-Thr-Ser-Ser, PPL92.CHO: Met-Ala-Leu-Asn-Ser-Cys-Asn*-Ser-Thr-Ser (where * is the glycosylation site). After import and cleavage, the resulting polypeptides, PL62.Con (apparent molecular mass 7 kD) and PL62.CHO (apparent molecular mass 9 kD), have potential SMCC reactive groups at residues 4, 11, and 58 (Cys) and residues 42 and 48 (Lys), in addition to the free amino group at the NH_2-terminus.
12. Antisera recognizing ERp57, the heavy chain–binding protein (BiP), and a 55-kD ER calcium-binding protein (ERC-55) were tested. Of these, only ERp57 was found to be cross-linked to PL62.CHO and no cross-linking products with PL62.Con were detected.
13. ERp57 is also known as GRP58, ERp61, ER-60, Q-2, and HIP-70 [C. F. Bennett, J. M. Balcarek, A. Varrichio, S. T. Crooke, *Nature* **334**, 268 (1988); S. P. Srivastava, N. Chen, Y. Liu, J. L. Holtzman, *J. Biol. Chem.* **266**, 20337 (1991); N. Hirano *et al.*, *Biochem. Biophys. Res. Commun.* **204**, 375 (1994)].
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16. Dog pancreas microsomes were solubilized in 1% (w/v) saponin and centrifuged at 100,000*g*, and the supernatant was applied to a concanavalin A–Sepharose column to remove glycoproteins. The unbound material was precipitated with $(NH_4)_2SO_4$, dialyzed against 10 mM tris-HCl (pH 7.5), and applied to a Resource Q column (Pharmacia). Bound proteins were eluted with a 0 to 1 M NaCl gradient; ERp57 eluted as a single protein and was identified by its molecular weight and by NH_2-terminal amino acid sequencing. The purified protein was then used to raise a rabbit polyclonal antibody. The resulting anti-ERp57 only functions for immunoprecipitation after SDS denaturation (Fig. 1B); thus, a reciprocal coimmunoprecipitation experiment (Fig. 2B) using anti-ERp57 in the first round was not possible.
17. After import of *S. cerevisiae* prepro–α factor cDNA (in pGEM) into microsomes, the resulting protein has three sites for N-linked glycosylation and nine lysine residues from which cross-linking may occur. Human IFN-γ has two sites for N-linked glycosylation, one cysteine residue, and 20 lysine residues. The transcription vector used was as described [N. J. Bulleid, E. Curling, R.B. Freedman, N. Jenkins, *Biochem. J.* **268**, 777 (1990)].
18. When the addition of SMCC to isolated microsomes was delayed for increasing lengths of time (*19*), the efficiency of cross-linking between PL62.CHO and ER components decreased. No loss of nascent PL62.CHO was observed over the same period. The estimated half-life for the PL62.CHO cross-linking products was 60 min for adducts with calnexin, calreticulin, and associated ERp57, and >120 min for the adduct with PDI.
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22. Cell-free translation was carried out under reducing conditions, protein synthesis was inhibited by the addition of emetine to a final concentration of 2 mM, and the membranes were isolated by centrifugation. The membranes were resuspended in LS buffer [250 mM sucrose, 100 mM KOAc, 5 mM $Mg(OAc)_2$, and 50 mM Hepes-KOH (pH 7.9)], which permits the oxidation of full-length prolactin, and the samples were treated with puromycin for 5 min. SMCC was added to a final concentration of 1 mM from a 50 mM stock in DMSO; samples were incubated for 10 min and then quenched by addition of 0.1 volumes of 50 mM 2-mercaptoethanol and 500 mM glycine. All reactions were carried out at the translation temperature of 26°C.
23. Rabbit antibodies to canine ERp57 were purified from crude serum by affinity purification using protein immobilized on nitrocellulose [E. Harlow and D. Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988)].
24. All of the glycosylated forms indicated in Fig. 3D were sensitive to digestion with endoglycosidase H. After import, signal sequence cleavage, and glycosylation, the major forms of PPα and IFN-γ had apparent molecular masses of 29 and 25 kD, respectively.

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Early Onset of Reproductive Function in Normal Female Mice Treated with Leptin

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Numerous studies have revealed an association between nutritional status, adiposity, and reproductive maturity. The role of leptin, a hormone secreted from adipose tissue, in the onset of reproductive function was investigated. Normal prepubertal female mice injected with leptin grew at a slower rate than controls as a result of the hormone's thinning effects, but they reproduced up to 9 days earlier than controls and showed earlier maturation of the reproductive tract. These results suggest that leptin acts as a signal triggering puberty, thus supporting the hypothesis that fat accumulation enhances maturation of the reproductive tract.

A link between body fat content and the onset of puberty in females was first proposed over 30 years ago (*1*, *2*). More recent studies documenting delayed puberty in lean female ballet dancers (*3*, *4*) and accelerated puberty in obese females (*5*) support the concept that a metabolic signal produced by adipose tissue may control the onset of reproductive function (*6*). The ability of leptin, a hormone secreted by adipose tissue, to restore fertility to mice that are genetically deficient in leptin (*7*) suggests that this hormone may be a signal triggering the onset of reproductive function.

To explore this possibility, we injected human recombinant leptin into normal prepubertal female mice and monitored its circulatory levels over time (*8*). Leptin had a

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